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A Renaissance in Understanding the Multiple and Diverse Functions of Granzymes?

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In this issue of *Immunity*, Metkar et al. (2008) present evidence that granzyme A plays a role in inflammatory signaling and that contrary to previous studies, it is incapable of inducing target cell death. The work challenges us to reconsider the broader biological roles of all the granzymes.

Together with the pore-forming protein perforin, the family of granule-bound serine proteases known as granzymes forms an antiviral arsenal central to the function of cytotoxic T lymphocyte (CTL) and natural killer (NK) cells. The prevailing view is that perforin facilitates entry of granzymes into the cytoplasm of a target cell, where they access their substrates to trigger cell death (Voskoboinik et al., 2006). Humans and rodents have three granzyme subfamilies encoded on distinct chromosomal loci: (1) granzymes A and K have trypsin-like activity; (2) granzyme M cleaves after unbranched hydrophobic residues; and (3) human granzymes B and H and rodent granzymes B through G have chymotrypsin-like activity. Although there is no doubt that granzyme B plays an important role in inducing apoptosis, and it is generally accepted that granzyme A can trigger a distinct nonapoptotic form of cell death, the cytotoxicity of other granzymes is less certain. Over the years, a number of other functions have been suggested for granzymes, but few studies have dealt with these topics, particularly of late.

Although a molecular or cellular mechanism is not comprehensively defined, Metkar et al. (2008) now show that granzyme A secreted by CTLs promotes the release of IL-1β and other inflammatory cytokines from antigen-presenting cells such as primary mouse macrophages in vitro. It is inferred from the data that granzyme A enters activated monocytes or macrophages to potently induce inflammatory cytokine release. Release can be blocked with a caspase-1 inhibitor, suggesting that the production of active IL-1β does not result from direct processing of pro-IL-1β by granzyme A. The in vivo relevance of these findings is indicated by the fact that mice deficient for granzyme A are partially resistant to lipopolysaccharide (LPS)-induced toxic shock, which is known to be mediated by the release of proinflammatory cytokines including IL-1β. However, these results seem to be confounded by the unexplained and conflicting observations that granzyme B-deficient mice are also resistant to LPS, whereas granzyme A and B doubly-deficient mice are as sensitive as wild-type. It is difficult to reconcile these results, and we are also left to ponder whether granzyme-mediated inflammation is perforin dependent.

A key question posed by Metkar et al. (2008) is whether granzyme A plays any significant role in target cell death. The investigators use a variety of assays but fail to reproduce the cytotoxic activity of granzyme A other than at very high concentrations, thereby challenging the view that cell-death induction is an important and intrinsic function of this protease. To put these findings into context, Hayes et al. (1989) were the first to describe synergy between granzymes and perforin leading to apoptosis. Soon after, three different DNA-fragmenting activities within rat CTL granules were identified as granzyme A (fragmentin-1), granzyme B (fragmentin-2), and granzyme K (fragmentin-3) (Shi et al., 1992). Further elegant studies demonstrated that expression of perforin in RBL-2H3 cells enables killing via necrosis, but that coexpression with granzymes

results in apoptosis (Nakajima et al., 1995). Over the next decade, many laboratories elaborated the mechanisms leading to DNA fragmentation and death induced by granzyme B, and the group headed by J. Lieberman progressively characterized a distinct caspase-independent cell-death mechanism mediated by human granzyme A.

In contrast to the studies of Shi et al. (1992), the Lieberman group found that vigorous single-stranded DNA nicking rather than oligonucleosomal DNA fragmentation is the end-point of the granzyme A death pathway. To explain this discrepancy, Metkar et al. (2008) postulate that inadvertent granzyme B contamination of granzyme A preparations caused the observed DNA fragmenting activity. In the Lieberman model, granzyme A directly penetrates the mitochondrial matrix and disrupts the electron transport chain through cleavage of components of complex 1. How granzyme A accesses the matrix is unknown, because it lacks a mitochondrial targeting sequence. This important problem aside, the induction of reactive oxygen species production is proposed to cause translocation of the SET complex into the nucleus. Coincident cleavage of SET proteins by granzyme A results in single-stranded DNase activity that rapidly causes cell death (see Martinvalet et al., 2008).

How can we reconcile the findings of Metkar et al. (2008) with the Lieberman model (Martinvalet et al., 2008)? In support of Metkar et al. (2008), we have also failed to confirm cell-death-inducing activity of human granzyme A in vitro, whereas that of mouse granzyme A was at least an order of magnitude weaker than human granzyme B (Kaiserman et al., 2006). In vivo, granzyme A-deficient mice have no defect of cytolysis or DNA fragmentation, but the cytotoxic defect for mice lacking both granzymes A and B can be greater (depending on the target cell) than for granzyme B-deficient mice. Furthermore, expression of mouse granzyme A in RBL cells increases the efficiency of perforin-mediated death (Nakajima et al., 1995). These data indicate that mouse granzyme A is cytotoxic and can facilitate or amplify cell death caused by other cytotoxins. Mice and humans possess a second "tryptase" (granzyme K), so functional redundancy may mask a defect in programmed cell death in granzyme A-deficient mice. This possibility will be excluded once mice deficient for both granzymes are produced.

Significant species-specific differences in the substrate specificity of granzymes have recently been found (Kaiserman et al., 2006), and thus it is likely that confusion in the field has arisen through comparison of granzymes, perforins, and cytotoxic lymphocytes of different species, or through mixing components from different species in the same in vitro experiment. For instance, the Lieberman group has largely used human granzyme A in its biochemical studies, whereas those working on biological functions have used mouse granzyme A or mice lacking it. An additional major caveat in vitro is that nobody knows the effective concentration at which granzymes or perforin are delivered to target cells at an authentic immunological synapse, and this remains a major impediment to determining how much granzyme is needed to kill a given target cell. Typically, low nanomolar concentrations of granzyme B and perforin potently induce apoptosis, whereas cell death caused by other granzymes (including granzyme A) requires 500 to 2000 nM. It may therefore be possible that applying high concentrations of granzymes to cells can result in nonphysiological death, and that using micromolar concentrations of protease on cell extracts may result in cleavage of irrelevant substrates. On the first point, Metkar et al. (2008) demonstrate that addition of micromolar concentrations of active or inactive granzyme A with perforin causes membrane damage and necrosis, and we note that the Lieberman group routinely uses micromolar granzyme A in cytotoxicity assays. On the second point, caution should be applied when searching for substrates in cell-free systems, as illustrated by a recent proteomics study that identified hundreds of substrates when 200 nM granzyme B was applied to cell extracts, but only a handful when granzyme B was delivered by a NK cell (Van Damme et al., 2008). Such an approach will be necessary to finally determine whether SET complex components or inflammatory mediators are true targets of granzyme A. Weighing all of the above considerations, one way to reconcile the Metkar et al. (2008) and the Lieberman group findings is to suggest that human granzyme A is not cytotoxic unless applied at excessive concentrations, whereas mouse granzyme A possesses weak cell-death-promoting activity. This does not rule out the targeting of SET complexes by human granzyme A, but suggests that it is insufficient to cause cell death. By contrast, both human and mouse granzyme A seem capable of initiating a proinflammatory response.

The work of Metkar et al. (2008) forces us to reassess the physiological relevance of various proposed granzyme functions. The fact that granzyme A-deficient mice exhibit no apoptotic phenotype but are somewhat resistant to LPS-induced toxic shock points to the importance of granzyme A in promoting proinflammatory pathways preferentially over those that lead to cell death. In our view, four criteria make it clear that granzyme B is uniquely and powerfully proapoptotic: (1) caspase-like specificity, (2) potency at low nanomolar concentrations, (3) classic apoptotic cell-death morphology, and (4) the apoptosis-defective phenotype of granzyme B-deficient mice. None of the other granzymes fulfill these criteria, but researchers proposing cytotoxic roles for them might quite reasonably suggest that they mobilize diverse cell-death pathways to protect the host against viruses expressing a granzyme B inhibitor. However, this can also be achieved by directly targeting viral proteins. Indeed, granzyme H directly cleaves two adenoviral proteins: L4-100K, a capsid protein that inhibits granzyme B-induced apoptosis, and a DNA-binding protein that is vital for viral replication (Andrade et al., 2007).

To summarize, re-evaluation of old and new data leads us to propose that granzyme B is the prime inducer of cell death, whereas the other granzymes carry out alternative functions including activation of inflammatory pathways through pro-IL-1β processing (Figure 1). Thus, granzyme B is functionally equivalent to the proapoptotic caspases, whereas other granzymes mimic inflammatory caspases. Both cell-death signaling and pro-IL-1β processing occur in the cytoplasm, so a critical issue is whether the proinflammatory actions of granzymes require perforin, as has been established for granzyme B-induced apoptosis. The study of Metkar et al. (2008) will no doubt engender further interest in the

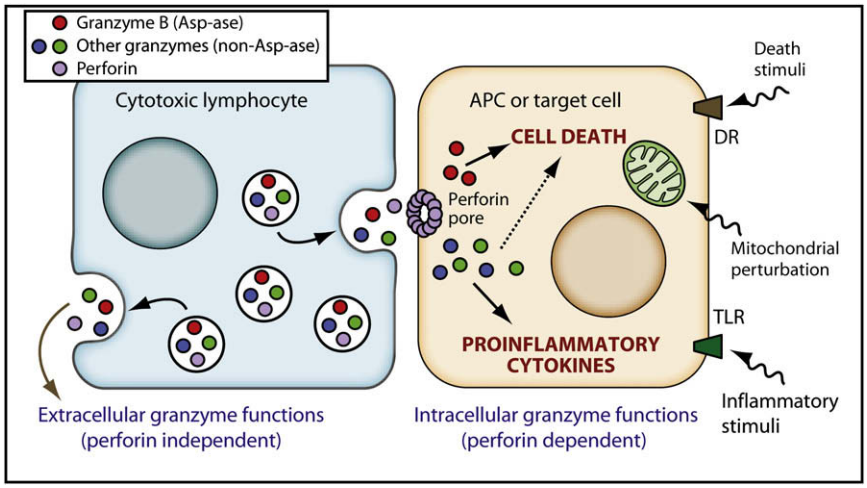

Figure 1. Granzymes: Extrinsic Activators of Cell Death and Inflammation that Mimic Intrinsic Caspase Pathways

Granzyme B, which like the proapoptotic caspases cleaves after specific Asp residues, is transferred from a cytotoxic lymphocyte into the cytoplasm of a target or antigen-presenting cell to impose death in response to virus infection or malignant transformation. Granzyme B (red dots) mediates this process in a perforin-dependent manner, as perforin (purple dots) provides access to its cytoplasmic substrates. The principal function of other (non-Asp-ase) granzymes (green and blue dots) within the target cell may be to mimic the proinflammatory caspases that are critical for inflammasome formation and release of cytokines such as IL-1β, IL-6, and TNF. It is presumed that this function of granzymes is also perforin dependent but this remains to be formally demonstrated. Other intrinsic cell-death and proinflammatory pathways can be activated by stimuli other than the granzymes, for example inflammatory stimuli operating through Toll-like receptors (TLR), cell death through ligation of death receptors (DR), or mitochondrial perturbation. In some situations, it might also be possible for granzymes other than B to activate target cell death, but far less efficiently than granzyme B. Other granzyme functions (cell detachment, chemotaxis, lymphocyte migration through the extracellular matrix) occur in the extracellular space and are independent of perforin.

nonapoptotic roles of granzymes, in accord with the prescient predictions made by Kramer and Simon (1987) more than twenty years ago.

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An Innate Path to Human B Cell Tolerance

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Self-reactive B cells are eliminated during development by antibody-affinity selection and receptor-editing mechanisms. Work by Isnardi et al. (2008) in this issue of *Immunity* suggests that removal of autoreactivity from the immature B cell pool also requires innate immunity pathways.

The random nature of antibody diversification processes guarantees that a large number of newly generated antibodies will recognize self-antigens. These potentially harmful antibodies with self-specificities are eliminated in large numbers at various steps of B cell development so that the mature B cell repertoire of a healthy individual is largely devoid of self-reactive antibodies. Knowledge of this process has greatly advanced ever since the Nussenzweig group developed a combination of single-cell polymerase chain reaction (PCR) and antibody-cloning techniques to investigate single B cell specificities in humans (Wardemann and Nussenzweig, 2007). This technique is now utilized by Isnardi et al. to characterize alterations in the naive B cell repertoire of patients with deficiencies in innate immune pathways (Isnardi et al., 2008).

The analysis of B cells from healthy individuals detects several checkpoints against autoreactive B cells in bone